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Date of Signature and Deposit: ~~December 9, 2003~~ Feb. 11, 2004

Nicholas J. Scay

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: James A. Thomson

Date: December 9, 2003

Serial No.: 09/522,030

Group Art Unit: 1632

Filed: 03/09/2000

Examiner: Joseph T. Woitach

For: SERUM FREE CULTIVATION OF PRIMATE EMBRYONIC STEM CELLS

File No.: 960296.96544

DECLARATION UNDER 35 C.F.R. §1.132

Commissioner For Patents
P O 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Tenneille Ludwig, on oath declare and say that:

1. I am employed as a Research Associate at the Primate Research Center at the University of Wisconsin-Madison. As a part of my research responsibilities, I investigate and work with conditions for culturing primate and human embryonic stem cells under the direction of Dr. James Thomson, who I understand is the inventor of the above-identified patent application.

2. I have been told that a question with regard to the above-identified patent application is whether the culture condition described in the patent applications are unique to the "KnockOut" Serum Replacer Product, identified as used in examples in that patent application, or whether that effect is more generalized to other formulations which include a mixture of similar components, but are not the same identical product. As it happens, one of my research

responsibilities is to identify and develop conditions for the culture of primate and human embryonic stem cells in which all of the constituents of the medium are as well defined as we can make them. As a part of that effort I have been investigating mixtures of components which can substitute for commercial products known as Serum Replacers. In that work, we use both human and primate embryonic stem cells.

3. We currently use in our laboratory a serum replacement product that we make ourselves. Informally, in our laboratory, we refer to this mixture as the TeSR Serum Replacer. The name of the product, TeSR, is actually the first two letters of my first name and the letters S and R for serum replacer. As we formulate TeSR Serum Replacer, the formulation consists of Albumax™ or an alternate purified albumin, vitamins, minerals, antioxidants, insulin, transferrin and lipids.

4. I conducted a series of experiments designed to evaluate the suitability of the TeSR Serum Replacement as a substitute for the commercial Serum Replacer of the type described in the specification of the above-identified patent application. Experiments were performed using both rhesus and human embryonic stem cells. In conducting these tests, I used a stem cell culture medium that consisted of a balanced salt nutrient mixture base medium, (DMEM for rhesus cells and DMEM/DF12 for the human studies), which respectively comprised 80% of the final formulation. The remaining 20% of the medium was either the commercial serum replacement product, or the TeSR Serum Replacer. Both media were then supplemented with glutamine, ß-mercaptoethanol, non-essential amino acids (NEAA), and basic fibroblast growth factor (bFGF), all as previously described.

5. For studies using rhesus cells, identical numbers of rhesus embryonic stem cells originating from a single culture were plated into two different treatments. In one culture the treatment medium consisted of 80% DMEM plus 20% of the commercial serum replacer, plus the supplements listed above. This was our standard or control since we know this formulation can be used to maintain stem cells in culture. The experimental medium consisted of 80% DMEM plus 20% TeSR and the supplements.

6. Cells in each treatment were cultured on Matrigel™ matrix with medium that had

been conditioned by being exposed overnight to mouse embryonic fibroblasts plated at a density of 2.12×10^5 cells per milliliter and maintained at 37°C in a humidified atmosphere of 5% carbon dioxide in air. Cells were regularly passed when they reached confluence, which occurred roughly every five to seven days. The culture continued for nine passages at which point the experiment was terminated.

7. The result of the above-described experiment was that after nine passages there was no notable difference in growth between the primate embryonic stem cells cultured in standard embryonic stem cell medium or those cultured in the modified TeSR containing medium. The embryonic stem cells cultured at similar rates and were morphologically consistent across treatments with known morphologies of embryonic stem cells remaining and proliferating in an undifferentiated state.

8. For studies using the human cells, identical numbers of human embryonic stem cells originating from a single culture were plated into two different treatments. In one culture, the treatment medium consisted of 80% DMEM/DF12 plus 20% of the commercial serum replacement product and the supplements. This was our standard or control, since we know that this formulation can be used to maintain and proliferate human embryonic stem cells in an undifferentiated state. The experimental medium consisted of 80% DMEM/DF12 plus 20% of the TeSR and the same supplements.

9. Cells in each treatment were cultured directly on mouse embryonic fibroblasts plated at a density of 0.75×10^5 cells per milliliter and maintained at 37°C in humidified atmosphere of 5% carbon dioxide in air. Cells were regularly passaged when they reached confluence, which occurred roughly once every five to seven days. The experiment was performed in triplicate. The culture continued for five passages at which point the experiment was terminated.

10. The result of the above-described experiment was that after five passages there was no significant difference in growth between the human embryonic stem cell cultured in standard embryonic stem cell medium or those cultured in the modified TeSR containing medium. The embryonic stem cells cultured in the two treatments cultured at similar rates (cells in the

modified TeSR containing medium passaged one day later than those in the control medium) and were morphologically consistent across treatments with known morphologies of embryonic stem cell cultures remaining and proliferating in an undifferentiated state.

11. From these experiments, I conclude that the TeSR is an effective alternative to serum for the maintenance of proliferation of both human and non-human primate embryonic stem cells and seems to contain everything necessary for the growth of primate embryonic stem cells when combined with fibroblasts growth factor and the other supplements mentioned above. The constituents of the TeSR medium are sufficient, with the other constituents mentioned above, to culture primate embryonic stem cells in an undifferentiated state grown on fibroblast feeder cells or an alternate matrix if the medium is first conditioned on MEFs.

12. I hereby declare all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and the such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated this 9th day of December, 2003

Tenneille Ludwig

Tenneille Ludwig

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